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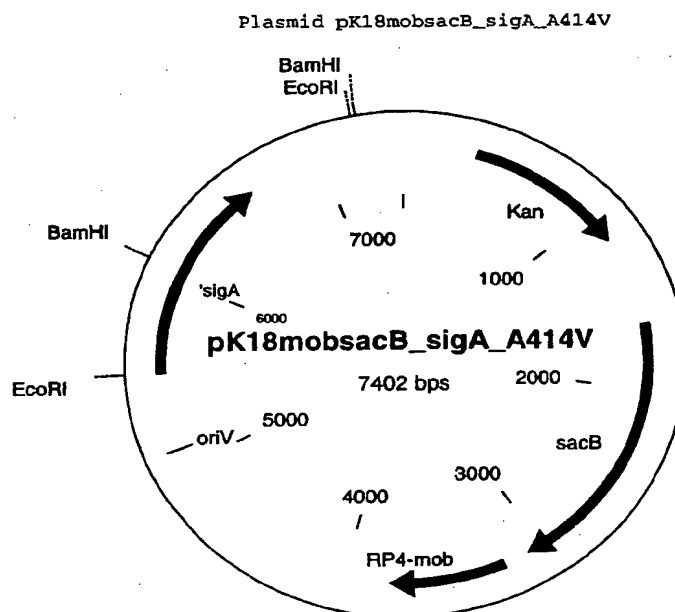
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(54) Title: ALLELES OF THE SIGA GENE FROM CORYNEFORM BACTERIA



(57) Abstract: The invention relates to alleles of the sigA gene from coryneform bacteria which code for sigma factors A and a process for the fermentative preparation of L-lysine using bacteria which contain these alleles.

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## Alleles of the sigA gene from coryneform bacteria

### Field of the Invention

The invention provides alleles of the sigA gene from coryneform bacteria which code for variants of sigma factor A and a process for the fermentative preparation of L-lysine using bacteria which contain these alleles.

### Prior art

The amino acid L-lysine is used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and very particularly in animal nutrition.

It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can relate to fermentation measures, such as, for example, stirring and supply of oxygen, or the composition of the nutrient media, such as, for example, the sugar concentration during the fermentation, or the working up to the product form by, for example, ion exchange chromatography, or the intrinsic output properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites or are auxotrophic for metabolites of regulatory importance and which produce amino acids are obtained in this manner. A known antimetabolite is the lysine analogue S-(2-aminoethyl)-L-cysteine (AEC).

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of *Corynebacterium* strains which produce L-amino acid, by

amplifying individual amino acid biosynthesis genes and investigating the effect on the amino acid production.

The nucleotide sequence of the gene which codes for sigma factor A from *Corynebacterium glutamicum* can be found in  
5 the patent application EP-A-1108790 as sequence no. 2100 and as sequence no. 7065.

The nucleotide sequence is also deposited in the databank of the National Center for Biotechnology Information (NCBI) of the National Library of Medicine (Bethesda, MD, USA)  
10 under Accession Number AX122184 and under Accession Number AX127149.

Object of the invention

The inventors had the object of providing new measures for improved fermentative preparation of L-lysine.

#### 15 Summary of the invention

When L-lysine or lysine are mentioned in the following, not only the bases but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are meant by this.

The invention provides replicatable nucleotide sequences  
20 (DNA) which originate from coryneform bacteria, in particular *Corynebacterium glutamicum*, and code for sigma factor A, wherein the associated amino acid sequences in SEQ ID No. 2 contains any proteinogenic amino acid excluding L-alanine at position 414.

25 The invention furthermore provides a replicatable nucleotide sequence (DNA) which originates from coryneform bacteria, in particular *Corynebacterium glutamicum*, and codes for sigma factor A, wherein the associated amino acid sequence contains L-valine at position 414, shown in SEQ ID  
30 No. 4.

The invention furthermore provides a replicatable nucleotide sequence (DNA) which originates from coryneform bacteria, in particular *Corynebacterium glutamicum*, and codes for sigma factor A, the base sequence of which  
5 contains thymine at position 1241, shown in SEQ ID No. 3.

The invention furthermore provides plasmids (vectors) which contain the nucleotide sequences according to the invention and optionally replicate in coryneform bacteria.

The invention furthermore provides coryneform bacteria  
10 which contain the nucleotide sequences according to the invention and in which the nucleotide sequences which code for sigma factor A are optionally present in over-expressed form, wherein the associated amino acid sequences contain another proteinogenic amino acid at position 414 of SEQ ID  
15 No. 2.

Over-expression is understood as meaning an increase in the intracellular concentration or activity of the sigma factors A according to the invention.

By over-expression measures, the activity or concentration  
20 of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on the activity or concentration of the protein in the starting microorganism.

25 Sigma factor A is a transcription factor which mediates the binding of RNA polymerase to specific sites (initiation sites) of the DNA and initiates the start (initiation) of transcription. It participates in the initiation of transcription a large number of genes, for example the  
30 genes *hom*, which codes for homoserine dehydrogenase, *gap*, which codes for glyceraldehyde 3-phosphate dehydrogenase, *fda*, which codes for fructose bisphosphate aldolase, and

pgk, which codes for phosphoglycerate kinase (Pátek et al., Microbiology 143: 1297-1309 (1996)).

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative L-lysine production. The expression is likewise improved by measures to prolong the life of the mRNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Plasmids which are replicated in coryneform bacteria are suitable for increasing the number of copies of the sigma alleles according to the invention. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEX1 (Eikmanns et al., Gene 102:93-98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as e.g. those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A 5,158,891) can be used in the same manner.

The method of chromosomal gene amplification, such as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)) for duplication or amplification of the hom-thrB operon, can

furthermore be used to increase the number of copies. In this method, the complete gene or allele is cloned in a plasmid vector which can replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman, Journal of Biological Chemistry 269:32678-84 (1994); US-A 5,487,993), pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)), pEM1 (Schrumpf et al., Journal of Bacteriology 173:4510-4516 (1991)) or pBGS8 (Spratt et al., Gene 41: 337-342 (1986)). The plasmid vector which contains the gene or allele to be amplified is then transferred into the desired strain of *C. glutamicum* by conjugation or transformation. The method of conjugation is described, for example, by Schäfer et al. (Applied and Environmental Microbiology 60, 756-759 (1994)). Methods for transformation are described, for example, by Thierbach et al. (Applied Microbiology and Biotechnology 29, 356-362 (1988)), Dunican and Shivnan (Bio/Technology 7, 1067-1070 (1989)) and Tauch et al. (FEMS Microbiological Letters 123, 343-347 (1994)). After homologous recombination by means of a "cross over" event, the resulting strain contains at least two copies of the gene or allele in question.

The increase in protein concentration is detectable via 1- and 2-dimensional protein gel separation and subsequent optical identification of the protein concentration in the gel with appropriate evaluation software. A common method for preparation of the protein gels in the case of coryneform bacteria and for identification of the proteins is the procedure described by Hermann et al. (Electrophoresis, 22:1712-23 (2001)). The protein concentration can also be analysed by western blot hybridization with an antibody specific for the protein to be detected (Sambrook et al., Molecular cloning: a

laboratory manual. 2<sup>nd</sup> Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989) and subsequent optical evaluation with appropriate software for determination of the concentration (Lohaus and Meyer (1998) Biospektrum 5:32-39; Lottspeich (1999) Angewandte Chemie 111:2630-2647). The activity of DNA-binding proteins can be measured by means of DNA band shift assays (also called gel retardation) (Wilson et al. (2001) Journal of Bacteriology 183:2151-2155). The effect of DNA-binding proteins on the expression of other genes can be detected by various well-described methods of reporter gene assay (Sambrook et al., Molecular cloning: a laboratory manual. 2<sup>nd</sup> Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989).

The invention provides replicatable, preferably endogenous nucleotide sequences (DNA) which originate from coryneform bacteria and code for the protein sigma factor A, wherein in the associated amino acid sequences the L-alanine at position 414 of SEQ ID No. 2 is replaced by another proteinogenic amino acid, in particular L-valine, shown in SEQ ID No. 4.

The invention also provides replicatable, preferably endogenous nucleotide sequences (DNA) which originate from coryneform bacteria and code for the protein sigma factor A, the associated base sequence of which contains thymine at position 1241, shown in SEQ ID No. 3.

"Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences present in the population of a species.

The invention also provides vectors (plasmids) which contain the nucleotide sequences mentioned and optionally replicate in coryneform bacteria.

Coryneform bacteria which preferably contain the nucleotide sequence(s) mentioned according to the nucleotide sequences



which code for sigma factor A in an over-expressed form are also claimed.

The invention provides a process for the preparation of L-lysine or feedstuffs additives comprising L-lysine in which  
5 in general the following steps are carried out:

- a) fermentation of coryneform bacteria which contain endogenous nucleotide sequences which code for sigma factor A, wherein in the associated amino acid sequences the L-alanine at position 414 is replaced by  
10 another proteinogenic amino acid, preferably L-valine, the alleles of the endogenous sigA gene are over-expressed under conditions suitable for the formation of the sigA gene product sigma factor A,
- b) concentration of the L-lysine in the fermentation  
15 broth,
- c) isolation of the L-lysine or feedstuffs additive comprising L-lysine from the fermentation broth, optionally
- d) with constituents from the fermentation broth and/or  
20 the biomass (> 0 to 100%).

Proteinogenic amino acids are to be understood as meaning all amino acids which are constituents of proteins or polypeptides. These are, in particular: L-aspartic acid, L-asparagine, L-threonine, L-serine, L-glutamic acid, L-  
25 glutamine, glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan, L-proline and L-arginine.

The wild-type form of the sigA gene is contained in wild-  
30 type strains of coryneform bacteria, in particular of the

genus corynebacterium. It is shown in SEQ ID No. 1. The wild-type protein is shown in SEQ ID No. 2.

Of the genus Corynebacterium, the species Corynebacterium glutamicum known to experts is to be mentioned in particular. Known wild-type strains of the species  
5 Corynebacterium glutamicum are, for example

Corynebacterium glutamicum ATCC13032  
Corynebacterium acetoglutamicum ATCC15806  
Corynebacterium acetoacidophilum ATCC13870  
10 Corynebacterium melassecola ATCC17965  
Corynebacterium thermoaminogenes FERM BP-1539  
Brevibacterium flavum ATCC14067  
Brevibacterium lactofermentum ATCC13869 and  
Brevibacterium divaricatum ATCC14020.

15 Strains with the designation "ATCC" can be obtained from the American Type Culture Collection (Manassas, VA, USA). Strains with the designation "FERM" can be obtained from the National Institute of Advanced Industrial Science and Technology (AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba  
20 Ibaraki, Japan). The strain of Corynebacterium thermoaminogenes mentioned (FERM BP-1539) and others (FERM BP-1540, FERM BP-1541 and FERM BP-1542) are described in US-A 5,250,434.

To produce the sigA alleles according to the invention  
25 which code for variants of sigma factor A characterized by an amino acid exchange at position 414 of SEQ ID No. 2, mutagenesis methods described in the prior art are used.

Conventional in vivo mutagenesis methods using mutagenic substances, such as, for example, N-methyl-N'-nitro-N-  
30 nitrosoguanidine, or ultraviolet light can be used for the mutagenesis.

In vitro methods, such as, for example, a treatment with hydroxylamine (Miller, J. H.: A Short Course in Bacterial

Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1992) or mutagenic oligonucleotides (T. A. Brown: Gentechnologie für  
5 Einsteiger [Genetic Engineering for Beginners], Spektrum Akademischer Verlag, Heidelberg, 1993) or the polymerase chain reaction (PCR) such as is described in the handbook by Newton and Graham (PCR, Spektrum Akademischer Verlag, Heidelberg, 1994) can furthermore be used for the  
10 mutagenesis.

Further instructions on generation of mutations can be found in the prior art and in known textbooks of genetics and molecular biology, such as e.g. the textbook by  
Knippers ("Molekulare Genetik", 6th edition, Georg Thieme  
15 Verlag, Stuttgart, Germany, 1995), that by Winnacker ("Gene und Klone", VCH Verlagsgesellschaft, Weinheim, Germany, 1990) or that by Hagemann ("Allgemeine Genetik", Gustav Fischer Verlag, Stuttgart, 1986).

If in vitro methods are used, the sigA gene described in  
20 the prior art is amplified starting from isolated complete DNA of a wild-type strain with the aid of the polymerase chain reaction, optionally cloned in suitable plasmid vectors, and the DNA is then subjected to the mutagenesis process. Instructions for amplification of DNA sequences  
25 with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994). Suitable  
30 sigA alleles are then selected by the processes described above and investigated.

The invention provides a new sigA allele which codes for a variant of sigma factor A and is shown in SEQ ID No. 3.

The sigA alleles according to the invention can be transferred into suitable strains by the method of gene replacement, such as is described by Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)) or Peters-Wendisch et al. (Microbiology 144, 915 - 927 (1998)). The corresponding sigA allele is cloned here in a vector which is not replicative for *C. glutamicum*, such as, for example, pK18mobsacB or pK19mobsacB (Jäger et al., Journal of Bacteriology 174: 5462-65 (1992)) or pCR®Blunt (Invitrogen, Groningen, Holland; Bernard et al., Journal of Molecular Biology, 234: 534-541 (1993)) and this is then transferred into the desired host of *C. glutamicum* by transformation or conjugation. After homologous recombination by means of a first "cross-over" event which effects integration and a suitable second "cross-over" event which effects excision in the target gene or in the target sequence, the incorporation of the mutation is achieved.

In addition, it may be advantageous for the production of L-amino acids at the same time to enhance, in particular over-express one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the use of the sigA allele according to the invention. The use of endogenous genes is in general preferred.

"Endogenous genes" or "endogenous nucleotide sequences" are understood as meaning the genes or nucleotide sequences and alleles thereof present in the population of a species.

The term "enhancement" in this connection describes the increase in the intracellular activity or concentration of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene or allele which codes for

a corresponding enzyme or protein having a high activity, and optionally combining these measures. An increase in the activity of the corresponding enzyme protein can also be effected by a reduced sensitivity to inhibitors.

- 5 By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the
- 10 activity or concentration of the protein in the starting microorganism.

Thus, for the preparation of L-lysine, in addition to the use of the variants of the sigA gene, at the same time one or more of the endogenous genes chosen from the group

15 consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology
- 20 174:6076-6086),
- the eno gene which codes for enolase (DE: 19947791.4),
- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase
- 25 (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661, EP-A-1108790),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609; EP-A1108790),

- the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No. P26512; EP-B-0387527; EP-A-0699759; WO 00/63388),
- the lyse gene which codes for the lysine export protein (DE-A-195 48 222),
- the zwal gene which codes for the Zwal protein (DE: 19959328,0, DSM 13115)
- the gnd gene which codes for 6-phosphogluconate dehydrogenase (WO 01/71012),
- the opcA gene which codes for a sub-unit of glucose 6-phosphate dehydrogenase (sequence no. 79 from WO 01/00844; WO 01/04322),

can be enhanced, in particular over-expressed.

The enhancement of 6-phosphogluconate dehydrogenase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-proline for L-serine, L-leucine, L-isoleucine or L-threonine at position 158 of the enzyme protein and/or by exchange of L-serine for L-phenylalanine or L-tyrosine at position 361 of the enzyme protein.

The enhancement of the glucose 6-phosphate dehydrogenase sub-unit can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-serine by L-phenylalanine or L-tyrosine at position 312 of the enzyme protein.

It may be furthermore advantageous for the production of L-lysine, in addition to the use of the variants of the sigA

gene, at the same time for one or more of the endogenous genes chosen from the group consisting of

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1, DSM 13047),
- 5 • the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- the poxB gene which codes for pyruvate oxidase (DE:1995 1975.7, DSM 13114),
- 10 • the zwa2 gene which codes for the Zwa2 protein (DE: 19959327,2, DSM 13113),
- the fda gene which codes for fructose 1,6-bisphosphate aldolase (Accession No. X17313; von der Osten et al., Molecular Microbiology 3 (11), 1625-1637 (1989)),
- 15 • the hom gene which codes for homoserine dehydrogenase (EP-A -0131171),
- the leuB gene which codes for isopropyl malate dehydrogenase (Pátek et al., Applied Environmental Microbiology 50:43-47(1989)), Accession No. Y09578),
- 20 • the leuC gene which codes for isopropyl malate dehydratase (Accession No. AX121536, sequence no. 1452 from patent EP1108790, Accession No. AX063983, sequence no. 265 from patent WO0100843),
- the thrB gene which codes for homoserine kinase (Peoples, O.W., et al., Molecular Microbiology 2:63-72(1988)) and
- 25 • the pfkB gene which codes for phosphofructokinase (SEQ ID No. 57 from WO 01/00844)

to be attenuated, in particular for the expression thereof to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes or proteins in a microorganism which are coded by the corresponding DNA, for example by using a weak promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme or protein, and optionally combining these measures.

By attenuation measures, the activity or concentration of the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

The attenuation of isopropyl malate dehydrogenase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-glycine for L-aspartate, L-asparagine or L-glutamate at position 131 of the enzyme protein.

The attenuation of isopropyl malate dehydratase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-arginine for L-serine at position 451 or L-glycine for L-aspartate at position 456 of the enzyme protein or a combination thereof.

The attenuation of homoserine dehydrogenase can also be achieved, inter alia, by amino acid exchanges, such as, for example, by exchange of L-asparagine for L-threonine or L-serine at position 118 or L-leucine for L-proline at position 160 of the enzyme protein or a combination thereof.

The attenuation of phosphofructokinase can also be achieved, inter alia, by amino acid exchanges, such as, for



example, by exchange of L-leucine for L-alanine, L-glycine or L-proline at position 109 of the enzyme protein.

- The invention also provides the microorganisms prepared according to the invention, and these can be cultured
- 5 continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of L-amino acids. A summary of known culture methods is described in the textbook by Chmiel
- 10 (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).
- 15 The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology
- 20 (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as, for example, soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as,

25 for example, palmitic acid, stearic acid and linoleic acid, alcohols, such as, for example, glycerol and ethanol, and organic acids, such as, for example, acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

- 30 Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be

used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

- Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the abovementioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.
- Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as, for example, fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, such as, for example, antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as, for example, air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.
- Methods for the determination of L-amino acids are known from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by anion exchange chromatography with subsequent ninhydrin derivatization, or it can be carried out by reversed phase HPLC, for example

as described by Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention is used for fermentative preparation of L-lysine.

- 5 The concentration of L-lysine can optionally be adjusted to the desired value by addition of L-lysine.

The present invention is explained in more detail in the following with the aid of embodiment examples.

Example 1

- 10 Amplification and sequencing of the DNA of the sigA allele of strain DM1547

- The *Corynebacterium glutamicum* strain DM1547 was prepared by multiple, non-directed mutagenesis, selection and mutant selection from *C. glutamicum* ATCC13032. The strain is  
15 resistant to the lysine analogue S-(2-aminoethyl)-L-cysteine and methionine-sensitive.

- From the strain DM1547, chromosomal DNA is isolated by the conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). With the aid of the polymerase chain  
20 reaction, a DNA section which carries the sigA gene or allele is amplified. On the basis of the sequence of the sigA gene known for *C. glutamicum* (sequence no. 2100 and sequence no. 7065 from EP1108970), the following primer oligonucleotides are chosen for the PCR:

- 25 sigA-1 (SEQ ID No. 8):  
5' tgatcggctgaccaactcta 3'

sigA-2 (SEQ ID No. 9):  
5' aaggtctcgaatccgagaac 3'

- The primers shown are synthesized by MWG Biotech  
30 (Ebersberg, Germany) and the PCR reaction is carried out by

the standard PCR method of Innis et al. (PCR protocols. A Guide to Methods and Applications, 1990, Academic Press). The primers allow amplification of a DNA section of approx. 1.89 kb in length, which carries the sigA allele.

- 5 The amplified DNA fragment of approx. 1.89 kb in length which carries the sigA allele of the strain DM1547 is identified by electrophoresis in a 0.8% agarose gel, isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).
- 10 The nucleotide sequence of the amplified DNA fragment or PCR product is determined by sequencing by MWG Biotech (Ebersberg, Germany). The sequence of the PCR product is shown in SEQ ID No. 5. The sequence of the coding region is shown again in SEQ ID No. 3. The amino acid sequences of  
15 the associated sigma factor A protein resulting with the aid of the Patentin program are shown in SEQ ID No. 6 and 4.

At position 1241 of the nucleotide sequence of the coding region of the sigA allele of strain DM1547, that is to say  
20 at position 1466 of the nucleotide sequence shown in SEQ ID No. 5, is the base thymine. At the corresponding position of the wild-type gene is the base cytosine (SEQ ID No. 1).

At position 414 of the amino acid sequence of sigma factor A of strain DM1547 is the amino acid valine (SEQ ID No. 6  
25 and 4). At the corresponding position of the wild-type protein is the amino acid alanine (SEQ ID No. 2).

The sigA allele, which contains the base thymine at position 1241 of the coding region and accordingly codes for a sigma factor A which contains the amino acid valine  
30 at position 414 of the amino acid sequence, is called the sigA\_A414V allele in the following. In the designation "sigA\_A414V", A represents L-alanine, V represents L-valine

and 414 indicates the position of the amino acid exchange (see SEQ ID No. 2 and 4).

### Example 2

Replacement of the sigA wild-type gene of strain DSM5715 by  
5 the sigA\_A414V allele

2.1. Production of a DNA fragment which carries the region of the sigA\_A414V allele on which the mutation A414V is located

From the strain DM1547, chromosomal DNA is isolated by the  
10 conventional methods (Eikmanns et al., Microbiology 140: 1817 - 1828 (1994)). A DNA section which carries the region of the sigA\_A414V allele on which the mutation A414V is located is amplified with the aid of the polymerase chain reaction. On the basis of the sequence of the sigA gene  
15 known for *C. glutamicum* (sequence no. 2100 and sequence no. 7065 from EP-A-1108790), the following primer oligonucleotides are chosen for the PCR such that the mutation A414V is located in the central region of the amplification product:

20 sigA\_XL-A1 (SEQ ID No. 10):  
5' ac gaa ttc-cga cgg cga tga ctt cgt ag 3'

sigA\_XL-A2 (SEQ ID No. 11):  
5' tg gaa ttc-cgt tcc acc tcg ctc cat tc 3'

The primers shown are synthesized by MWG Biotech  
25 (Ebersberg, Germany) and the PCR reaction is carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press). The primers allow amplification of a DNA section approx. 1.69 kb in length which carries a region of the sigA\_A414V  
30 allele (SEQ ID No. 7). The primers moreover contain the sequence for a cleavage site of the restriction

endonuclease EcoRI, which is marked by underlining in the nucleotide sequence shown above.

The amplified DNA fragment of approx. 1.69 kb in length which carries the sigA allele of the strain DM1547 is

5 cleaved with the restriction endonuclease EcoRI, identified by electrophoresis in a 0.8% agarose gel and then isolated from the gel and purified by conventional methods (QIAquick Gel Extraction Kit, Qiagen, Hilden).

## 2.2. Construction of the exchange vector

10 pK18mobsacB\_sigA\_A414V

The approx. 1.68 kb long DNA fragment cleaved with the restriction endonuclease EcoRI, which contains a region of the sigA\_A414V allele which carries the mutation A414V, is incorporated by means of replacement mutagenesis with the  
15 aid of the sacB system described by Schäfer et al. (Gene, 14, 69-73 (1994)) into the chromosome of the C. glutamicum strain DSM5715. This system enables preparation and selection of allele exchanges which take place by homologous recombination.

20 The mobilizable cloning vector pK18mobsacB is digested with the restriction enzyme EcoRI and the ends are dephosphorylated with alkaline phosphatase (Alkaline Phosphatase, Boehringer Mannheim, Germany). The vector prepared in this way is mixed with the sigA\_A414V fragment  
25 of approx. 1.68 kb and the mixture is treated with T4 DNA ligase (Amersham-Pharmacia, Freiburg, Germany).

The E. coli strain S17-1 (Simon et al., Bio/Technologie [Bio/Technology] 1: 784-791, 1993) is then transformed with the ligation batch (Hanahan, In. DNA cloning. A practical  
30 approach. Vol.1. ILR-Press, Cold Spring Harbor, New York, 1989). Selection of plasmid-carrying cells is made by plating out the transformation batch on LB agar (Sambrook et al., Molecular Cloning: a laboratory manual. 2<sup>nd</sup> Ed.

Cold Spring Harbor, New York, 1989), which was supplemented with 25 mg/l kanamycin.

Plasmid DNA is isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by  
5 restriction cleavage with the enzyme BamHI and subsequent agarose gel electrophoresis. The plasmid is called pK18mobsacB\_sigA\_A414V and is shown in figure 1.

### 2.3 Allele exchange

The vector pK18mobsacB\_sigA\_A414V mentioned in example 2.2  
10 is transferred by conjugation by a protocol of Schäfer et al. (Journal of Microbiology 172: 1663-1666 (1990)) into the *C. glutamicum* strain DSM5715. The vector cannot replicate independently in DSM5715 and is retained in the cell only if it is present integrated in the chromosome as  
15 the consequence of a recombination event. Selection of transconjugants, i.e. clones with integrated pK18mobsacB\_sigA\_A414V, is made by plating out the conjugation batch on LB agar (Sambrook et al., Molecular Cloning: a laboratory manual. 2<sup>nd</sup> Ed., Cold Spring Harbor,  
20 New York, 1989), which is supplemented with 15 mg/l kanamycin and 50 mg/l nalidixic acid. Kanamycin-resistant transconjugants are plated out on LB agar plates with 25 mg/l kanamycin and incubated for 24 hours at 33°C. For  
25 selection of mutants in which excision of the plasmid has taken place as a consequence of a second recombination event, the clones are cultured unselectively for 30 hours in LB liquid medium and then plated out on LB agar with 10% sucrose and incubated for 16 hours.

The plasmid pK18mobsacB\_sigA\_A414V, like the starting  
30 plasmid pK18mobsacB, contains, in addition to the kanamycin resistance gene, a copy of the sacB gene which codes for levan sucrase from *Bacillus subtilis*. The expression which can be induced by sucrose leads to the formation of levan sucrase, which catalyses the synthesis of the product

levan, which is toxic to *C. glutamicum*. Only those clones in which the integrated pK18mobsacB\_sigA\_A414V has excised as the consequence of a second recombination event therefore grow on LB agar. Depending on the position of the second recombination event with respect to the mutation site, allele exchange or incorporation of the mutation takes place with the excision, or the original copy remains in the chromosome of the host.

Approximately 40 to 50 colonies are tested for the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin". In 4 colonies which show the phenotype "growth in the presence of sucrose" and "non-growth in the presence of kanamycin", a region of the sigA gene spanning the mutation A414V is sequenced, starting from the sequencing primer sA\_1 (SEQ ID No. 12), by GATC Biotech AG (Constance, Germany) to demonstrate that the mutation of the sigA\_A414V allele is present in the chromosome. The primer sA\_1 used is synthesized for this by GATC:

sA\_1 (SEQ ID No. 12):  
5' aag ttc tcc acc tac gca ac 3'

A clone which contains the base thymine at position 1241 of the sigA gene and thus has the sigA\_A414V allele was identified in this manner. This clone was called strain DSM5715sigA\_A414V.

### Example 3

#### Preparation of lysine

The *C. glutamicum* strain DSM5715sigA\_A414V obtained in example 2 is cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant is determined.



For this, the strain is first incubated on an agar plate for 24 hours at 33°C. Starting from this agar plate culture, a preculture is seeded (10 ml medium in a 100 ml conical flask). The medium MM is used as the medium for the  
5 preculture. The preculture is incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture is seeded from this preculture such that the initial OD (660 nm) of the main culture is 0.1. The Medium MM is also used for the main culture.

10

## Medium MM

CSL	5 g/l
MOPS	20 g/l
Glucose (autoclaved separately)	50 g/l
Salts:	
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	25 g/l
KH <sub>2</sub> PO <sub>4</sub>	0.1 g/l
MgSO <sub>4</sub> * 7 H <sub>2</sub> O	1.0 g/l
CaCl <sub>2</sub> * 2 H <sub>2</sub> O	10 mg/l
FeSO <sub>4</sub> * 7 H <sub>2</sub> O	10 mg/l
MnSO <sub>4</sub> * H <sub>2</sub> O	5.0mg/l
Biotin (sterile-filtered)	0.3 mg/l
Thiamine * HCl (sterile-filtered)	0.2 mg/l
L-Leucine (sterile-filtered)	0.1 g/l
CaCO <sub>3</sub>	25 g/l

The CSL (corn steep liquor), MOPS (morpholinopropanesulfonic acid) and the salt solution are

brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions, as well as the CaCO<sub>3</sub> autoclaved in the dry state, are then added.

5 Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Culturing is carried out at 33°C and 80% atmospheric humidity.

After 72 hours, the OD is determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The amount of lysine formed is  
10 determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in table 1.

Tabelle 1

Strain	OD (660 nm)	Lysine HCl g/l
DSM5715	8.2	13.57
DSM5715sigA_A414V	8.0	15.21

15

Brief description of the figure:

Figure 1: Map of the plasmid pK18mobsacB\_sigA\_A414V.

The abbreviations and designations used have the following meaning. The base pair numbers stated are approximate  
20 values obtained in the context of reproducibility of measurements.

Kan: Kanamycin resistance gene

EcoRI: Cleavage site of the restriction enzyme  
EcoRI

BamHI: Cleavage site of the restriction enzyme  
BamHI

'sigA: Cloned DNA fragment containing a 3' terminal  
region of the sigA allele (= sigA\_A414V  
allele) and the downstream region

sacB: sacB gene

RP4-mob: mob region with the replication origin for  
the transfer (oriT)

oriV: Replication origin V

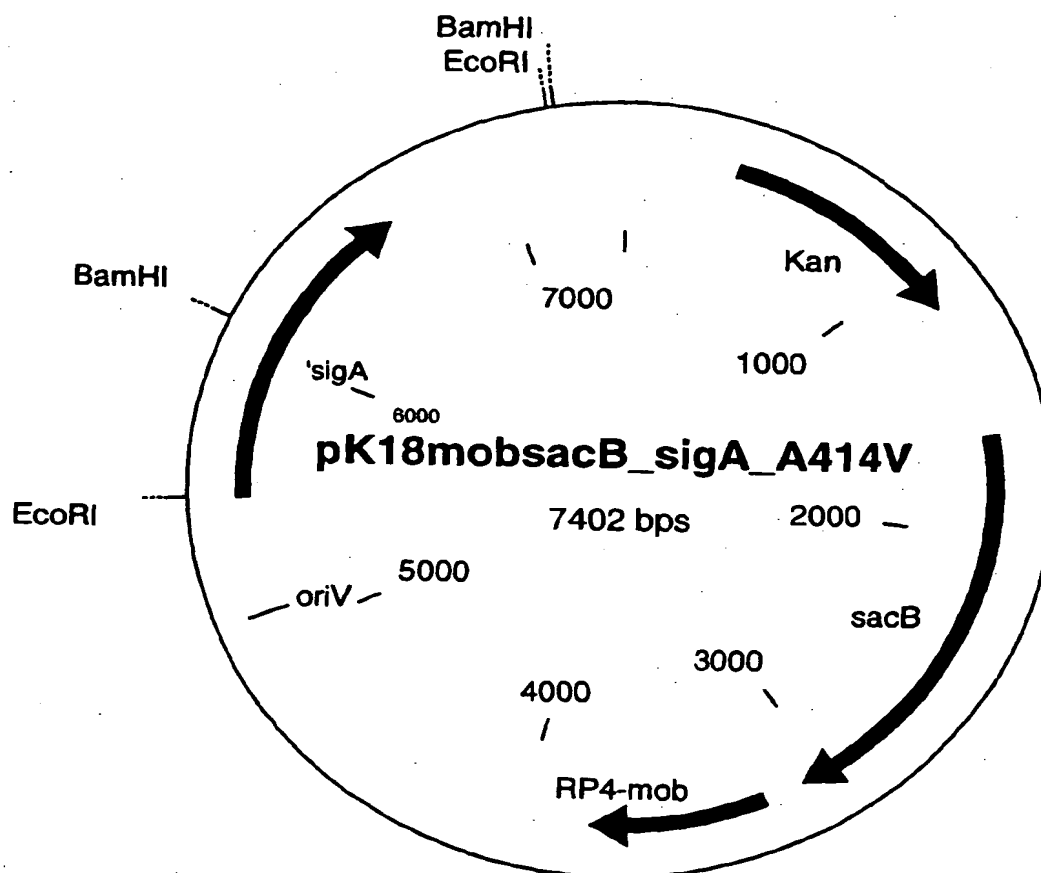
**WHAT IS CLAIMED IS:**

1. Replicable nucleotide sequences (DNA) which originate from coryneform bacteria and code for the protein sigma factor A, wherein in the associated amino acid sequences the L-alanine at position 414 of SEQ ID No. 2 is replaced by another proteinogenic amino acid.
2. Replicable nucleotide sequence (DNA) according to claim 1 which originates from coryneform bacteria and codes for the protein sigma factor A, wherein the associated amino acid sequence contains the L-valine at position 414, shown in SEQ ID No. 4.
3. Replicable nucleotide sequence (DNA) according to claim 1 which originates from coryneform bacteria and codes for the protein sigma factor A, the base sequence of which contains thymine at position 1241, shown in SEQ ID No. 3.
4. Plasmids (vectors) which contain the nucleotide sequences according to claims 1 to 3 and optionally replicate in coryneform bacteria.
5. Coryneform bacteria which contain nucleotide sequences according to claims 1 to 4 and in which the nucleotide sequences which code for sigma factor A are preferably present in over-expressed form.
6. Coryneform bacteria which contain nucleotide sequences which code for sigma factor A, wherein in the associated amino acid sequences the L-alanine at position 414 of SEQ ID No. 2 is replaced by another proteinogenic amino acid.
7. Process for the preparation of L-lysine or feedstuffs additives comprising L-lysine, in which the following steps are carried out (comprising):

- 5           a)    fermentation of coryneform bacteria in which  
              alleles of the endogenous sigA gene are over-  
              expressed under conditions which are suitable for  
              the formation of the sigA gene product sigma  
              factor A; and
- b)    isolation of the L-lysine or the feedstuffs  
                      additive comprising L-lysine from the  
                      fermentation broth, the coryneform bacteria  
                      forming the L-lysine.
- 10   8.    Process according to claim 7, wherein coryneform  
          bacteria which contain an allele of the sigA gene are  
          employed, wherein in the associated amino acid  
          sequences the L-alanine at position 414 of SEQ ID No.  
          2 is replaced by another proteinogenic amino acid.
- 15   9.    Process according to claim 7, wherein microorganisms  
          in which further genes of the biosynthesis pathway of  
          L-lysine are additionally over-expressed are employed.
10. Process according to claim 7, wherein microorganisms  
              in which the metabolic pathways which reduce the  
20            formation of L-lysine are at least partly eliminated  
              are employed.
11. Process for the preparation of L-lysine or feedstuffs  
              additives comprising L-lysine, in which the following  
              steps are carried out:
- 25           a)    fermentation of coryneform bacteria which contain  
                      endogenous nucleotide sequences which code for the  
                      protein sigma factor A, wherein in the associated  
                      amino acid sequences the L-alanine at position 414  
                      is replaced by another proteinogenic amino acid,  
30            preferably L-valine,
- b)    concentration of the L-lysine in the fermentation  
                      broth,

- c) isolation of the L-lysine or feedstuffs additive comprising L-lysine from the fermentation broth, optionally
  - d) with constituents from the fermentation broth and/or the biomass (> 0 to 100%).
- 5

Figure 1: Plasmid pK18mobsacB\_sigA\_A414V



## SEQUENCE LISTING

&lt;110&gt; Degussa AG

&lt;120&gt; Alleles of the sigA gene from coryneform bacteria

&lt;130&gt; 010481 BT

&lt;160&gt; 12

&lt;170&gt; PatentIn version 3.1

&lt;210&gt; 1

&lt;211&gt; 1497

&lt;212&gt; DNA

&lt;213&gt; Corynebacterium glutamicum

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1)..(1494)

&lt;223&gt; sigA wild-type gene

&lt;400&gt; 1

gtg	gag	agc	agc	atg	gta	gaa	aac	aac	gta	gca	aaa	aag	acg	gtc	gct	48
Met	Glu	Ser	Ser	Met	Val	Glu	Asn	Asn	Val	Ala	Lys	Lys	Thr	Val	Ala	
1				5					10					15		

aaa	aag	acc	gca	cgc	aag	acc	gca	cgc	aaa	gca	gcc	ccg	cgc	gtg	gca	96
Lys	Lys	Thr	Ala	Arg	Lys	Thr	Ala	Arg	Lys	Ala	Ala	Pro	Arg	Val	Ala	
			20					25					30			

acc	cca	ttg	gga	gtc	gca	tct	gag	tct	ccc	att	tcg	gcc	acc	cct	gcg	144
Thr	Pro	Leu	Gly	Val	Ala	Ser	Glu	Ser	Pro	Ile	Ser	Ala	Thr	Pro	Ala	
		35					40					45				

cgc	agc	atc	gat	gga	acc	tca	acc	cct	gtt	gaa	gct	gct	gac	acc	ata	192
Arg	Ser	Ile	Asp	Gly	Thr	Ser	Thr	Pro	Val	Glu	Ala	Ala	Asp	Thr	Ile	
	50					55					60					

gag	acc	acc	gcc	cct	gca	gcg	aag	gct	cct	gcg	gcc	aag	gct	ccc	gct	240
Glu	Thr	Thr	Ala	Pro	Ala	Ala	Lys	Ala	Pro	Ala	Ala	Lys	Ala	Pro	Ala	
65					70					75				80		

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Lys	Lys	Val	Ala	Lys	Lys	Thr	Ala	Arg	Lys	Ala	Pro	Ala	Lys	Lys	Thr	
			85						90					95		

gtc	gcc	aag	aaa	gcc	aca	acc	gcc	aag	gct	gca	cct	gca	act	gcc	aag	336
Val	Ala	Lys	Lys	Ala	Thr	Thr	Ala	Lys	Ala	Ala	Pro	Ala	Thr	Ala	Lys	
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gac	gaa	aac	gca	cct	gtt	gat	gac	gac	gag	gag	aac	ctc	gct	cag	gat	384
Asp	Glu	Asn	Ala	Pro	Val	Asp	Asp	Asp	Glu	Glu	Asn	Leu	Ala	Gln	Asp	
		115					120					125				

gaa	cag	gac	ttc	gac	ggc	gat	gac	ttc	gta	gac	ggc	atc	gaa	gac	gaa	432
Glu	Gln	Asp	Phe	Asp	Gly	Asp	Asp	Phe	Val	Asp	Gly	Ile	Glu	Asp	Glu	
		130				135					140					



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ctg tcc aaa gaa atg gac atc tcc gag gaa aag gta ctg gaa atc cag Leu Ser Lys Glu Met Asp Ile Ser Glu Glu Lys Val Leu Glu Ile Gln 370 375 380	1152

cag tac gcc cgc gaa cca atc tcc ctg gac caa acc atc ggc gac gaa 1200  
 Gln Tyr Ala Arg Glu Pro Ile Ser Leu Asp Gln Thr Ile Gly Asp Glu  
 385 390 395 400  
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 Gly Asp Ser Gln Leu Gly Asp Phe Ile Glu Asp Ser Glu Ala Val Val  
 405 410 415  
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 65 70 75 80  
 Lys Lys Val Ala Lys Lys Thr Ala Arg Lys Ala Pro Ala Lys Lys Thr  
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 Gln Glu Val Ser Leu Ala Lys Arg Ile Glu Ala Gly Leu Tyr Ala Thr  
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 His Arg Met Glu Glu Met Glu Glu Ala Phe Ala Ala Gly Asp Lys Asp  
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 245 250 255  
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 Ala Val Asp Ala Val Ser Phe Thr Leu Leu Gln Asp Gln Leu Gln Asp  
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aaa aag acc gca cgc aag acc gca cgc aaa gca gcc ccg cgc gtg gca 96  
Lys Lys Thr Ala Arg Lys Thr Ala Arg Lys Ala Ala Pro Arg Val Ala  
20 25 30  
acc cca ttg gga gtc gca tct gag tct ccc att tcg gcc acc cct gcg 144  
Thr Pro Leu Gly Val Ala Ser Glu Ser Pro Ile Ser Ala Thr Pro Ala  
35 40 45  
cgc agc atc gat gga acc tca acc cct gtt gaa gct gct gac acc ata 192  
Arg Ser Ile Asp Gly Thr Ser Thr Pro Val Glu Ala Ala Asp Thr Ile  
50 55 60  
gag acc acc gcc cct gca gcg aag gct cct gcg gcc aag gct ccc gct 240  
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65 70 75 80  
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Lys Lys Val Ala Lys Lys Thr Ala Arg Lys Ala Pro Ala Lys Lys Thr  
85 90 95  
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115 120 125  
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Val Glu Ser Ser  
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Arg Lys Thr Ala Arg Lys Ala Ala Pro Arg Val Ala Thr Pro Leu Gly  
25 30 35  
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Val Ala Ser Glu Ser Pro Ile Ser Ala Thr Pro Ala Arg Ser Ile Asp  
40 45 50  
gga acc tca acc cct gtt gaa gct gct gac acc ata gag acc acc gcc 429  
Gly Thr Ser Thr Pro Val Glu Ala Ala Asp Thr Ile Glu Thr Thr Ala  
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cct gca gcg aag gct cct gcg gcc aag gct ccc gct aaa aag gtt gcc 477  
Pro Ala Ala Lys Ala Pro Ala Ala Lys Ala Pro Ala Lys Lys Val Ala  
70 75 80  
aag aag aca gct cgc aag gca cct gcg aaa aag act gtc gcc aag aaa 525  
Lys Lys Thr Ala Arg Lys Ala Pro Ala Lys Lys Thr Val Ala Lys Lys  
85 90 95 100



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 485 490 495  
 Leu Asp

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 <213> Artificial sequence

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 the downstream region

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 <221> mutation  
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 <223> Exchange of cytosine for thymine

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PCT/EP 02/11561

International Application No.  
PCT/EP 02/11561

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 C12N9/12 C12N15/31 C12N15/52 C07K14/34 C07K14/345  
C12N15/77 C12N1/21 C12P13/08

According to International Patent Classification (IPC) or to both national classification and IPC

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 1 108 790 A (KYOWA HAKKO KOGYO KK) 20 June 2001 (2001-06-20) ----	
A	WO 01 66573 A (BASF AG) 13 September 2001 (2001-09-13) ----	
A	HALGASOVA NORA ET AL: "Cloning and transcriptional characterization of two sigma factor genes, sigA and sigB, from <i>Brevibacterium flavum</i> ." CURRENT MICROBIOLOGY, vol. 43, no. 4, October 2001 (2001-10), pages 249-254, XP002227863 ISSN: 0343-8651 ----- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

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- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "A" document member of the same patent family

Date of the actual completion of the international search

20 January 2003

Date of mailing of the international search report

05/02/2003

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Authorized officer

Marinoni, J-C

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 02/11561

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	<p>OGUIZA J A ET AL: "Multiple sigma factor genes in brevibacterium lactofermentum: characterization of sigA and sigB"  JOURNAL OF BACTERIOLOGY, WASHINGTON, DC, US,  vol. 178, no. 2, January 1996 (1996-01),  pages 550-553, XP002183859  ISSN: 0021-9193</p>	



# INTERNATIONAL SEARCH REPORT

Information on patent family members

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